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Implementing nickel phytomining in a serpentine quarry in NW Spain

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ABSTRACT

In Galicia (NW Spain), ultramafic outcrops represent approximately 5% of the land surface and several mining and quarrying activities take place in these areas. Resulting mine-soils present physical, chemical and biological properties which limit plant growth and soil functioning. Nickel phytomining, an eco-friendly strategy for metal recovery, could potentially be applied to these areas. A one-year field experiment was carried out in a serpentine quarry to evaluate the performance of four Ni hyperaccumulating plant species, comparing the Mediterranean spp. *Bornmuellera emarginata* and *Odontarrhena muralis* with the native populations of *Noccaea caerulescens* and *Odontarrhena serpyllifolia*. Field plots were established and amended with inorganic NPK fertilisers or composted sewage sludge. Three replicate plots (4 m^2) were planted for each plant species and fertilisation regime. Amending with compost reduced pH from 7.8 to 6.6, and increased soil cation exchange capacity (CEC), nutrient concentrations and Ni availability. Moreover, compost-amended mine-soil presented higher microbial density and activity, parameters which were further stimulated by plant growth. Plant biomass production of all plant species was significantly higher in compost-amended soils than that after NPK fertilisation, being most pronounced for *O. muralis* and *B. emarginata*. Despite the reduction in shoot Ni concentrations observed in plants (except *O. muralis*) grown in compost-amended plots, the increased biomass production led to significantly higher Ni yields (in kg ha^{-1}) in *B. emarginata* (2.9), *N. caerulescens* (1.9) and *O. muralis* (2.3). All plant species were able to establish and grow in the mine-soil (with the Mediterranean species showing a higher capacity for adaptation) and to generate moderate Ni yields. Nonetheless, the results highlight the need for further optimisation in order to enhance the Ni phytoextraction efficiency. Finally, the improvement in soil quality after compost amendment and plant growth support the idea that phytomining systems can be effective approaches for the rehabilitation of soils affected by quarrying operations after mine closure.

1. Introduction

Mining and quarrying activities exist since pre-historic times but the demand for raw materials has experienced an unprecedented growth since the early 20th century alongside an increasing global population and the development of new and existing technologies. The European Innovation Partnership (EIP) classified Ni as a raw material with high economic importance (EC, 2017). Traditional pyro- and hydro-metallurgical processes for nickel extraction require high energy and chemical inputs, and are usually performed on sulphide (and laterite) ores with elevated concentration(s) of the target metal(s). However, these ores are not widely distributed and for many elements, such as nickel, available reserves are gradually diminishing (Dalvi et al., 2004; Sheoran et al., 2009). Moreover, mining and quarrying operations have significant negative impacts on the environment, leading to changes in the landscape topography and generating large amounts of waste (during extraction and beneficiation processes) with associated release of potentially toxic elements into their surroundings. Mine tailings, heaps or dumps can also indirectly result in air and water pollution (Lago-Vila et al., 2017).

As a result, the European Commission (EC), within the framework of the *Raw Materials Initiative*, encourages the development of new means of metal extraction and reuse, and supports the shift towards a circular economy to ensure the secure supply of raw materials while minimising waste production (Dodson et al., 2012). Amongst the identified possible actions, the EC highlights the recovery of valuable metals from

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secondary sources, including wastes generated by the mining industry (EC, 2018). Phytomining is an environmentally-friendly green technology for obtaining industrially relevant trace elements from sub-economic ore bodies (such as low-grade natural metal-enriched soils), degraded or contaminated land, or waste streams, which meets with the goals of the EC in terms of elemental sustainability (Dodson et al., 2012; Nkrumah et al., 2016).

Nickel phytomining cultivates hyperaccumulator plant species ("metal crops") which can accumulate high amounts of this metal in their aboveground tissues: the harvested Ni-rich plant biomass is incinerated to obtain an ash or "bio-ore" that can be processed to recover metallic Ni or Ni-products, such as ferronickel, Ni salts, or Ni catalysts (Barbaroux et al., 2012; Simonnot et al., 2018). Serpentine soils, formed during the alteration of ultramafic rocks, are of great interest for Ni phytomining since they represent important Ni reservoirs of the Earth's surface (Proctor and Woodell, 1975). Moreover, due to their particular geochemical properties and associated flora they constitute hotspots of plant biodiversity (Echevarria, 2018), and over 90% of known Ni hyperaccumulating plant species are endemic to these soils (Pollard et al., 2014; Reeves et al., 2018). Geographically, Ni hyperaccumulators belong to two groups: (1) the Mediterranean region and (2) tropical and subtropical areas (Reeves et al., 2018). The first group is mainly represented by the Brassicaceae family and most studies in this field have evaluated the Ni phytoextraction potential of the Mediterranean hyperaccumulators belonging to the *Odontarrhena* genus (syn. *Alyssum*), mainly *Odontarrhena muralis* and *O. corsica* (Bani et al., 2015a; Li et al., 2003). Other Brassicaceae recognised for their phytomining potential include *Bornmuellera emarginata* (syn. *Leptoplax emarginata*) (Rešetnik et al., 2013) and *B. tymphaea* (Chardot et al., 2005). Nickel phytomining field trials have been carried out in agricultural soils developed over ultramafic rocks in Pöjske (Albania), Galicia (NW Spain) and Oregon (USA), with promising results (Bani et al., 2015a, 2007; Li et al., 2003; Pardo et al., 2018). In contrast, field-scale evaluations in mine-affected areas are rare, with recent initiatives being restricted to tropical regions, such as New Caledonia (Losfeld et al., 2015), Sabah (Malaysia) and the Philippines (van der Ent et al., 2013). These studies aim to produce metal-enriched plant biomass for Ni recovery using tropical hyperaccumulating species, while at the same time contributing towards the rehabilitation of mine-affected areas.

In Galicia (NW Spain), serpentine outcrops represent approximately 5% of the land surface (about 1470 km^2), and several mining activities are dedicated to the extraction of rocks for industrial or ornamental use, generating large amounts of sterile material which is accumulated in spoil heaps and tailings (Lago-Vila et al., 2017; Rodríguez-Seijo et al., 2014). Implementing phytomining options in these serpentine quarries could provide an additional metal source to primary ores and at the same time reduce hazardous waste volumes and assist site restoration. However, the lack of soil structure, low water holding capacity, limited organic matter content and nutrient deficiency, which are typical characteristics of mine-soils, make these environments unfavourable for plant growth and development, even in the case of metal-tolerant hyperaccumulators (Ernst, 2005). Elevated concentrations of potentially phytotoxic metals (in addition to Ni), such as Cr or Co, as well as an alkaline pH, can further exasperate the plant-growth limiting factors of serpentine mine-soils. Since both biomass production and a high concentration of the desired element in the harvestable plant parts are key factors for the success of phytomining (Reeves et al., 2018), improving soil conditions will play a central role in the development of these strategies in mine-affected areas. The use of mature (stabilised) organic amendments resulted in a significant stimulation in plant growth and Ni phytoextraction capacity of Ni hyperaccumulators of the *Odontarrhena* and *Noccaea* genera in pot trials using both natural ultramafic soils (Álvarez-López et al., 2016b) and serpentine quarry tailings (Ghasemi et al., 2018a). However, there is a need to evaluate the potential benefits of these amendments at a field-scale.

In this study the feasibility of Ni phytomining in serpentine mine spoils was assessed using four Ni hyperaccumulating species (*Bornmuellera emarginata*, *Noccaea caerulescens*, *Odontarrhena muralis* and *O. serpyllifolia*). In addition, the effects of incorporating composted sewage sludge as soil amendment on plant growth, biomass production and Ni bioaccumulation of the four hyperaccumulators was compared with inorganic NPK fertilisation.

2. Material and methods

2.1. Study site

The field experiment was carried out in a serpentine quarry in Bandeira (N 42°45′48″ N, S 8°17′00″) (Pontevedra, NW Spain). Bandeira is located in the Melide-Serra do Careón geological complex, which represents one of the three main serpentine outcrops of the Iberian Peninsula (Macías et al., 2009). It is characterised climatically by a high precipitation (annual mean 1375 mm) and mild temperatures (annual mean 12.6 °C). The active opencast mine is embedded in a substrate of amphibolites and serpentines, covering an area of 40 ha and is dedicated to the extraction of serpentinised peridotite for the production of gravel for construction and ballast for railway tracks. The generated sterile material is accumulated in spoil heaps and these cover an area of around 3.4 km^2 (Fig. 1). The soil derived from the spoil material – classified as Spolic Technosol (IUSS Working Group WRB, 2014) - is shallow and gravelly, with a poor structure, low water retention capacity and is mostly bare of vegetation, with *Cortaderia selloana* as the only successful colonising plant species.

The physico-chemical mine-soil characteristics given in Table 1 are in accordance with the geological complex of the area. The mine-soil is characterised by a basic pH (7.8) and poor fertility, which is reflected in the low total C and N content and nutrient concentrations (available P and K). Furthermore, the mine-soils are characterised by elevated concentrations of metals such as Ni (2302 mg kg^{-1}), Cr (1379 mg kg^{-1}) and Co (84 mg kg^{-1}). On the other hand, although the pseudo-total Mg concentration is much higher than that observed for Ca - which is typical of ultramafic soils, Ca dominates the cation exchange complex, thus maintaining a balanced Ca to Mg relation (Ca/Mg = 1.0; (Table 1).

2.2. Plant species and experimental design

Four Ni hyperaccumulating plant species were selected for this study: *Odontarrhena serpyllifolia* (Desf.) Jord & Fourr. (syn. *Alyssum serpyllifolium* subsp. *lusitanicum* T.R. Dudley & P.C. Silva) and *Noccaea caerulescens* (J. Presl & C. Presl) F.K. Mey, which are both found growing in the serpentine soils of the Iberian Peninsula, and *Odontarrhena muralis* (Waldst. & Kit). Endl. (syn. *Alyssum murale*) and *Bornmuellera emarginata* (Boiss.) Resetnik (syn. *Leptoplax emarginata* O.E. Schulz), which are native to Mediterranean serpentine soils. Seeds of *O. muralis* and *B. emarginata* were collected from the region of Trigona (Greece), *O. serpyllifolia* from Melide (NW Spain) and *Noccaea caerulescens* from Bandeira (NW Spain). Seeds were germinated in plastic trays (each with 81 plugs of 30 ml volume) filled with commercial potting substrate (Terrahumus) in a growth chamber under controlled conditions (temperature 22–25 °C, PPFD of 190 µmol m^{-2} s^{-1}, 16/8 h light/dark cycle) for two months before planting in the field.

In September 2016 an area of the spoils was cleared of vegetation, broken-up and tilled using a front loader tractor (the first 30–40 cm of surface soil). The experimental area was fenced-off and divided into two blocks, with a surface area of 105 m^2 each. One block was fertilised with inorganic NPK in the form of NH_4NO_3 (480 kg ha^{-1}) and KH_2PO_4 (400 kg ha^{-1}) and the other block was amended with composted sewage sludge at an addition rate of 210 t ha^{-1} (equivalent to 5% w/w). Addition rates were based on previous pot experiments (unpublished results). The compost was elaborated from sewage sludge and wood chippings by a local company (Tratamientos Ecológicos del

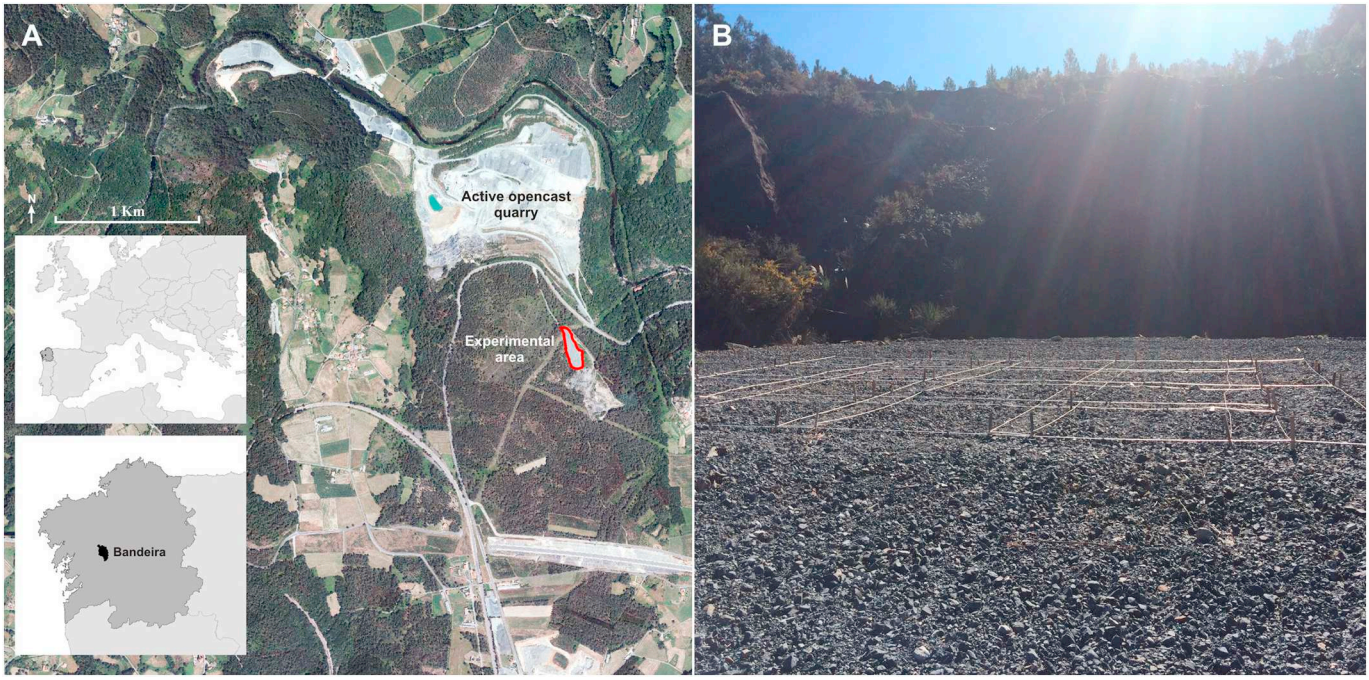

Fig. 1. A) Location of the serpentine quarry and, B) image of the experimental area before planting.

Table 1
Physico-chemical properties (mean ± SE) of the mine soil, the mine soil two months after the addition of composted sewage sludge (Mine soil + CSS) and the compost (CSS). Hyphens represent non-measured parameters.

	Mine soil	Mine soil + CSS	CSS
pH H_2O	7.8 ± 0.0	6.6 ± 0.0	6.0 ± 0.4
pH KCl	7.3 ± 0.0	6.1 ± 0.0	6.0 ± 0.0
P Olsen ($\mu g\,kg^{-1}$)	2.1 ± 0.4	240.8 ± 8.3	1704.0 ± 103.4
CEC ($cmol_c\,kg^{-1}$)	3.5 ± 0.3	19.2 ± 1.7	42.0 ± 4.3
Exchangeable Ca ($cmol_c\,kg^{-1}$)	1.8 ± 0.2	6.7 ± 0.4	11.7 ± 0.5
Exchangeable Mg ($cmol_c\,kg^{-1}$)	1.7 ± 0.1	9.6 ± 0.9	17.7 ± 0.8
Exchangeable K ($cmol_c\,kg^{-1}$)	0.1 ± 0.0	2.8 ± 0.4	12.6 ± 0.4
Ca/Mg	1.0 ± 0.1	0.7 ± 0.0	0.7 ± 0.1
% C	0.68 ± 0.01	6.03 ± 0.40	16.10 ± 0.20
% N	0.04 ± 0.01	0.59 ± 0.04	1.40 ± 0.01
C/N	13.7 ± 1.1	10.4 ± 0.0	12.0 ± 0.1
Hydrosoluble C ($mg\,l^{-1}$)	–	–	49.8 ± 7.1
Hydrosoluble N ($mg\,l^{-1}$)	–	–	52.4 ± 4.1
Pseudo-total element concentrations $g\,kg^{-1}$			
P	0.1 ± 0.0	0.4 ± 0.0	16.5 ± 0.3
K	0.2 ± 0.0	4.3 ± 0.0	8.9 ± 0.1
Al	8.0 ± 0.0	26.4 ± 0.0	31.3 ± 0.7
Ca	2.6 ± 0.0	5.2 ± 0.0	7.9 ± 0.1
Mg	216.8 ± 0.2	120.7 ± 0.1	10.4 ± 0.1
$mg\,kg^{-1}$			
Cr	1178.9 ± 12.1	719.3 ± 51.2	41.0 ± 1.4
Cu	11.3 ± 12.1	63.2 ± 3.4	168.4 ± 2.8
Mn	698.1 ± 6.0	606.7 ± 7.4	459.3 ± 1.4
Ni	1694.1 ± 14.4	977.2 ± 38.7	32.7 ± 0.1
Zn	20.7 ± 0.1	128.6 ± 5.9	311.5 ± 6.7

Noroeste, TEN s.l) and its principal physico-chemical properties are given in Table 1. The compost was quite fine (< 2 mm fraction representing 51% of mass), indicating a high accessibility of organic matter. The coarse fraction (> 2 mm) contributes to soil aeration. Assessments using transmission electronic microscopy (TEM) showed that the < 2 mm fraction was mainly composed by highly transformed organic matter (humified plant cell walls and bacterial residues) reflecting a high maturity (stable carbon). The coarse fraction (> 2 mm) was composed by ligneous residues, which was incompletely transformed and constitutes a source of labile carbon for soil microorganisms (unpublished results). The compost presented a slightly acidic pH, C/N ratio of 12, high CEC, and low to moderate concentration of the trace metals Cu, Mn, and Zn (Table 1).

Both blocks were divided into 16 plots of 4 m × 4 m, with a 1 m spacing between each of them. Three random replicate plots were established for each plant species in each block. *Odontarrhena serpyllifolia, O. muralis* and *B. emarginata* were planted at a density of 4 plants m^{-2}, and in the case of *N. caerulescens* planting density was 90 plants m^{-2}.

Three non-planted plots were established in both blocks to evaluate the effects of plant growth on soil physico- and bio-chemical properties. Plants were harvested after 10 months of growth in July 2017, and during the growth period weeds were manually removed.

2.3. Plant analysis

Plant biomass production, nutritional status and metal content were determined for each plant species and plot at harvest. Plants were divided into shoots and roots, washed with pressurised tap water followed by deionised water, and oven-dried at 40 °C to determine the dry weight (DW). Dried root and shoot material was ground and approximately 0.1 g of plant material was digested in a 2:1 concentrated HNO_3:HCl mixture on a hot plate at 130 °C. The concentration of P, K, Ca, Co, Cr, Fe, Mg, Mn and Ni in plant tissues was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, model Vista-PRO, Varian). To estimate the ability of plants to bioconcentrate Ni in their aboveground biomass the Bioconcentration Factor (BCF) was calculated as the ratio of the shoot Ni concentration and the total Ni concentration in the soil. The metal yield (total Ni phytoextracted) was calculated on the basis of shoot DW yields and shoot Ni concentration for each plant species.

2.4. Soil analyses

Both planted and unplanted soil samples were collected at harvest for physico-chemical and microbiological analyses. Composite planted soil samples were obtained by combining the soil around the root ball of each plant (discarding the original plug from the germination tray) per plot during harvesting. For physico-chemical analysis, one part of each composite soil sample was air-dried and sieved to < 2 mm. Soil pH was measured in H_2O and 0.1 M KCl at a 1:2.5 (w/v) ratio. Total C and N were determined by combustion with a CHN analyser (Model CHN-1000, LECO Corp., St Joseph, MI). Available P was determined colorimetrically following Olsen's extraction method (0.5 M $NaHCO_3$ adjusted to pH 8.2, 1:20 w/v). Cation exchange capacity (CEC) was estimated using 1 M NH_4Cl (1:20 w/v) as the sum of exchangeable cations Ca, Mg, Al, Na and K, determined by ICP-OES. Metal availability was assessed through DTPA-TEA extraction with 0.005 M Diethylene Triamine Pentaacetic Acid (DTPA), 0.01 M $CaCl_2$, and 0.1 M triethanolamine adjusted to pH 7.3 (Lindsay and Norvell, 1978) and measured by ICP-OES.

For soil microbiological analyses, the < 4 mm fraction of fresh composite soil samples (stored at +4 °C) was used. To determine the culturable bacterial density, 5 g of soil were shaken for 30 min in 45 ml sterile sodium hexametaphosphate solution (1%). Then, 5 ml of the supernatant were diluted in 10-fold series and plated onto solid 284 culture medium (Schlegel et al., 1961) supplemented with 100 μg ml^{-1} of the fungicide cycloheximide. Medium 284 contains (per litre): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2{\cdot}6H_2O$, 0.03 g $CaCl_2{\cdot}2H_2O$, 0.04 g Na_2HPO_4, 10 mL Fe (III)NH_4 citrate solution (containing 48 mg/100 ml) plus oligoelements (1.5 mg $FeSO_4{\cdot}7H_2O$, 0.3 mg H_3BO_4, 0.19 mg $CoCl_2{\cdot}H_2O$, 0.08 mg $ZnSO_4{\cdot}7H_2O$, 0.02 mg $CuSO_4{\cdot}5H_2O$, 0.036 mg $Na_2MoO{\cdot}2H_2O$) adjusted to pH 7. The medium was supplemented with a mixture of different carbon sources: lactate (0.7 g L^{-1}), glucose (0.5 g L^{-1}), fructose (0.5 g L^{-1}) and succinate (0.8 g L^{-1}). In order to determine the densities of culturable metal-tolerant bacteria, the same dilutions were plated on solid 284 medium supplemented with 2.5 mM Ni (added as $NiSO_4$ $6H_2O$) and cycloheximide. After 7 days of incubation at 28 °C, colony forming units were counted and calculated per gram DW soil (CFUs g^{-1} soil).

The community-level physiological profile (CLPP) of the microbial population was performed using Biolog™ ECO-plates. Each 96-well plate contains 31 different C substrates plus a water control (three replicate each) and the method is based on the ability of microorganisms to oxidise the different C sources thus obtaining an estimation of the microbial functional diversity. Each well was inoculated with a 150 μl aliquot of the 10^{-2} soil suspension and plates were incubated in the dark at 28 °C. Colour development in each well was recorded every 24 h during 7 days by reading the absorbance at 590 nm using a microplate reader (PowerWave XS2, BioTek Instruments, USA). For each reading time, raw absorbance data were corrected by subtracting the zero hour reading point and the absorbance value given by the control well. The average well colour development (AWCD) was calculated as the sum of the corrected well absorbance values, divided by the 31 carbon sources. Absorbance data were also subdivided into six substrate categories (carbohydrates, carboxylic acids, polymers, amino acids, amines and miscellaneous substrates) to obtain the substrate average well colour development (SAWCD). Substrate richness values (S) represent the number of oxidised substrates and the Shannon's diversity (H′) and evenness (J′) indices were calculated using the following formula:

$$H' = \sum p_i \log_2 p_i$$

$$J' = \frac{H'}{H_{max}}$$

where $H_{max} = H'/\log_2 S$ and p_i is the proportional colour development of the i well relative to the total colour development of all wells.

Fluorescein diacetate (FDA) activity was determined following the method described by Adam and Duncan (2001) to estimate the global soil heterotrophic microbial activity. Briefly, 4 ml of tris(hydroxymethyl)aminomethane (THAM; 100 mM) was added to 1 g soil and FDA (0.2% w/v) to start the reaction. After 1 h of incubation at 25 °C, soils were centrifuged (3 min × 2000 rpm) and the absorbance in the supernatant was measured at 290 nm (PowerWave XS2, BioTek Instruments, USA). Soils without FDA substrate were employed as a control.

2.5. Statistical analysis

Data were analysed using the IBM SPSS Statistics v.24.0 software. The effects of the fertilisation regime on plant and soil parameters were assessed using a Students *t*-test. In order to determine significant differences amongst plant species and soil parameters of planted and unplanted plots under the same fertilisation regime, a one-way analysis of variance (ANOVA) followed by a multiple comparison of means using the post-hoc Tukey-test for independent means was carried out. Normality of variances was tested by the Kolmogorov-Smirnov test and data were transformed to accomplish normality when necessary.

3. Results

3.1. Soil physico-chemical properties

Two months after incorporating the compost and before plantation, the mean pH of the mine-soil was reduced to 6.6 (Table 1) and an increase in available P, CEC, and C and N contents were recorded compared to unamended mine-soil. Total concentrations of the principal metals, Cr, Mn and Ni were significantly lower after compost incorporation, whereas concentrations of Cu and Zn (present in the compost) were higher (Table 1). In contrast, DTPA-extractable metal concentrations were increased in the amended mine-soil.

Table 2 shows the changes induced in soil physico-chemical properties by the four hyperaccumulating plant species after 10 months of growth in NPK-fertilised (NPK) and compost-amended (CSS) plots. In the NPK plots, the mean pH value in non-planted (NP) soil was 7.0 ± 0.0, while plant growth led to an increase in pH which was significant in the case of *O. muralis* and *O. serpyllifolia* ($p < 0.05$). In the CSS plots, soil pH was significantly lower in comparison to NPK, and there were no significant differences between planted and non-planted (NP) soils (mean pH ranged from 6.4–6.6). Soil C and N contents were significantly higher in compost-amended soils compared to

Table 2
General physico-chemical properties (mean ± SE) of the tailings soil in non-planted and planted sub-plots of the NPK fertilised and compost amended (CSS) plots. NP: non-planted, Be: *Bormuellera emarginata*, Nc: *Noccaea caerulescens*, Om: *Odontarrhena muralis*, Os: *Odontarrhena serpyllifolia*. Letters indicate significant differences between each plant species or non-planted sub-plots and asterisks significant differences between the type of fertilisation ($p < 0.05$).

	NPK					CSS				
	NP	Be	Nc	Om	Os	NP	Be	Nc	Om	Os
pH_{H2O}	7.0 ± 0.0 b*	7.3 ± 0.1 ab*	7.2 ± 0.1 b*	7.4 ± 0.0 a*	7.6 ± 0.0 a*	6.4 ± 0.0 a	6.4 ± 0.1 a	6.5 ± 0.1 a	6.6 ± 0.0 a	6.5 ± 0.0 a
pH_{KCl}	7.3 ± 0.0 a*	6.2 ± 0.2 b	6.5 ± 0.2 b*	7.0 ± 0.0 a*	7.0 ± 0.0 a*	6.0 ± 0.0 a	6.0 ± 0.1 a	6.0 ± 0.1 a	6.1 ± 0.0 a	6.1 ± 0.0 a
Total C (%)	0.36 ± 0.00 b*	0.63 ± 0.11 b*	1.86 ± 0.11 a*	0.87 ± 0.01 a*	0.68 ± 0.02 b*	3.59 ± 0.03 d	5.65 ± 0.38 c	8.28 ± 0.078 a	7.32 ± 0.40 b	5.30 ± 0.63 c
Total N (%)	0.06 ± 0.01 a*	0.04 ± 0.00 a*	0.06 ± 0.01 a*	0.05 ± 0.00 a*	0.04 ± 0.00 a*	0.33 ± 0.00 c	0.50 ± 0.06 b	0.58 ± 0.03 a	0.64 ± 0.08 a	0.46 ± 0.06 b
C/N	5.9 ± 0.8 c*	16.0 ± 3.3 b	32.3 ± 7.1 a*	14.4 ± 3.6 b	17.8 ± 1.6 b*	10.7 ± 0.2 b	11.3 ± 0.8 b	14.4 ± 0.6 a	11.4 ± 0.2 b	11.5 ± 0.2 b
P Olsen (mg kg^{-1})	27.6 ± 17.3 b*	29.3 ± 9.8 b*	25.3 ± 4.8 b*	46.3 ± 7.9 a*	18.3 ± 0.7 c*	152.9 ± 13.5 b	177.8 ± 32.0 b	120.2 ± 25.1 b	272.2 ± 25.1 a	263.4 ± 25.9 a
CEC ($cmol_c$ kg^{-1})	0.9 ± 0.1 c*	3.1 ± 0.6 *	8.2 ± 0.4 a	2.1 ± 0.4 c*	6.0 ± 4.8 b*	4.3 ± 0.4 c	11.8 ± 1.0 b	9.9 ± 0.7 b	9.1 ± 0.8 b	18.1 ± 1.7 a
Ca^{2+}	0.4 ± 0.1 c*	0.6 ± 0.0 c*	3.8 ± 0.6 a	1.0 ± 0.2 bc*	1.6 ± 0.2 b*	2.1 ± 0.2 c	4.8 ± 0.5 b	4.7 ± 0.5 b	3.9 ± 0.3 b	7.2 ± 0.6 a
K^+	0.0 ± 0.0 b*	0.1 ± 0.0 b*	0.2 ± 0.0 a*	0.1 ± 0.0 b*	0.2 ± 0.0 a*	0.3 ± 0.0 c	1.1 ± 0.1 b	0.8 ± 0.0 b	0.9 ± 0.1 b	1.7 ± 0.1 a
Mg^{2+}	0.4 ± 0.0 c*	2.4 ± 0.7 b*	4.1 ± 0.1 a	1.0 ± 0.2 bc*	4.3 ± 3.6 a*	1.9 ± 0.2 c	5.9 ± 0.4 b	4.2 ± 0.2 b	4.3 ± 0.3 b	9.1 ± 0.09 a
Ca/Mg	1.0 ± 0.0 a	0.3 ± 0.1 c*	0.7 ± 0.2 b*	0.9 ± 0.0 a	0.5 ± 0.2 bc*	1.1 ± 0.0 a	0.8 ± 0.0 b	1.1 ± 0.1 a	0.9 ± 0.0 b	0.8 ± 0.0 b
DTPA-extractable metal concentrations (mg kg^{-1})										
Cu	0.3 ± 0.0 b*	0.6 ± 0.3 a*	0.3 ± 0.1 b*	0.5 ± 0.0 a*	0.3 ± 0.0 b*	7.6 ± 0.1 d	16.6 ± 1.9 b	12.8 ± 0.4 c	18.9 ± 1.7 a	10.3 ± 2.3 c
Ni	3.8 ± 0.0 d*	9.6 ± 2.8 bc*	26.2 ± 15.1 a*	18.7 ± 1.7 b*	8.2 ± 2.2 c*	18.7 ± 0.3 c	34.6 ± 0.9 b	44.2 ± 4.3 a	32.1 ± 1.4 b	21.2 ± 4.7 c
Mn	2.2 ± 0.1 a*	3.5 ± 0.0 a*	0.2 ± 0.1 b*	0.2 ± 0.0 b*	0.1 ± 0.0 b*	15.1 ± 0.3 d	37.9 ± 5.5 b	34.2 ± 2.9 b	45.4 ± 6.5 a	21.2 ± 5.7 c
Zn	0.5 ± 0.0 a*	0.2 ± 0. b*	0.4 ± 0.0 a*	0.4 ± 0.0 a*	0.2 ± 0.0 b*	15.4 ± 0.2 d	29.6 ± 3.3 b	37.7 ± 4.3 a	37.4 ± 3.6 a	21.1 ± 4.2 c

NPK-fertilised soils. Moreover, at harvest the C and N content of planted soils was significantly higher than NP soil. This was observed in both CSS and NPK plots (except for N which was only higher in planted soils for CSS). In the CSS plots, a mean value of 3.59% C and 0.33% N was found in NP soil, whereas after plant growth these ranged from 5.30–8.28% C and from 0.46–0.64% N. In the NPK plots, the mean C content increased from 0.36% to 0.63–1.86%. In all cases, the highest values were found in soils planted with *O. muralis* and *N. caerulescens*. Plant growth also induced changes in P availability, and this was more pronounced in the CSS plots and after growth of *O. muralis* and *O. serpyllifolia*: extractable P concentrations were increased by 1.8- and 1.7-fold ($p < 0.05$). Extractable P concentrations were consistently lower in the NPK plots than in CSS, but again were significantly higher after cultivating *O. muralis* ($p < 0.05$). Compost addition also enhanced soil CEC: the mean CEC was 0.9 ± 0.1 $cmol_c$ kg^{-1} in NPK plots compared to 4.3 ± 0.4 $cmol_c$ kg^{-1} in CSS plots (data given for NP soils; $p < 0.05$). Plant growth led to further increases in CEC. In CSS plots, CEC was increased by 2.1-, 2.3-, 2.7- and 4.0-fold after the growth of *O. muralis*, *N. caerulescens*, *B. emarginata* and *O. serpyllifolia*, respectively ($p < 0.05$). This plant-induced effect was less evident in NPK plots, where only *N. caerulescens* and *O. serpyllifolia* significantly increased soil CEC ($p < 0.05$). In all cases the increase in CEC in planted soils was mainly a result of a plant-induced increase in exchangeable Ca and Mg (Table 2).

Trace metal availability (DTPA-extractable) was significantly higher in compost-amended plots than NPK-fertilised plots (Table 2). Mean DTPA-extractable Cu and Mn concentrations in NP-CSS soils were 26.5- and 33.3-fold higher than in the corresponding soils in the NPK plots (Table 2; $p < 0.05$). Plant growth led to further increases in DTPA-extractable Ni concentrations in both NPK and CSS plots: increments of 2.2- to 6.9-fold and 1.1- to 2.4-fold were observed in NPK and CSS plots, respectively ($p < 0.05$). This effect varied according to plant species, DTPA-extractable Ni concentrations were highest under *N. caerulescens* and lowest under *O. serpyllifolia* in both NPK and CSS plots. Similar plant-induced effects on Mn availability were observed with all plant species but only in the CSS plots; and in this case *O. muralis* had a stronger influence ($p < 0.05$). In contrast, in NPK plots DTPA-extractable Mn concentrations were significantly lower in planted soils than NP soils (except for *B. emarginata* where no effect was found).

3.2. Effect of soil amendment on plant survival and biomass production

After the 10-month growth period, plant survival differed amongst the four species and, with the exception of *B. emarginata*, it was slightly higher in NPK-fertilised plots. The % survival for *B. emarginata, N. caerulescens*, *O. muralis* and *O. serpyllifolia* was 42, 62, 63 and 40%, respectively, in NPK plots and 83, 59, 54 and 29%, respectively, in CSS plots. Fig. 2 shows the shoot dry weight (DW) yields of the four hyperaccumulators grown under the two fertilisation regimes. In NPK plots, biomass production (g DW $plant^{-1}$) of *B. emarginata*, *O. serpyllifolia* and *O. muralis* was similar; whereas the mean shoot DW yield for *N. caerulescens* was 12-fold lower than that obtained with the other three plant species.

Plant growth was significantly enhanced in the CSS plots compared to NPK plots, and this was observed for all plant species ($p < 0.05$). In this case, the shoot DW yields followed the order *O. muralis* > *B. emarginata* > *O. serpyllifolia* > *N. caerulescens*. The most pronounced effect of compost amendment on plant growth was observed for *O. muralis*: shoot DW yields increased from 7.4 ± 2.9 to 125.4 ± 27.6 g $plant^{-1}$, representing a 17-fold increase compared to plants grown in NPK plots ($p < 0.05$). Compost had an intermediate effect on *B. emarginata*, shoot DW yield was 6.1-fold higher than that obtained with inorganic fertilisation. Finally, the lowest stimulation in growth was observed for *O. serpyllifolia* and *N. caerulescens*: DW yields increased from 7.3 to 26.7 g $plant^{-1}$ (3.6-fold) and from 0.6 to 1.9 g $plant^{-1}$ (3.3-fold), respectively ($p < 0.05$). Finally, a high

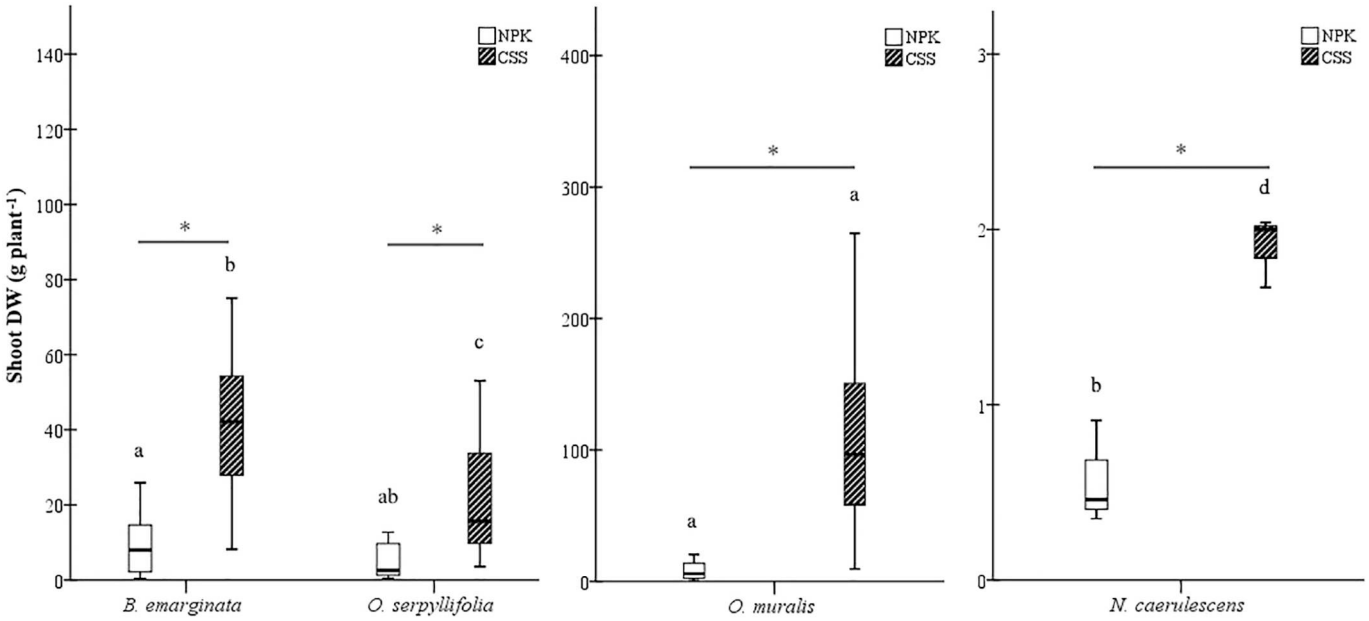

Fig. 2. Effect of fertilisation on the shoot biomass production (mean DW yield per plant ± SE) of each of the four hyperaccumulators. Median values are represented with a horizontal line within the boxes. Different letters above bars indicate significant differences between plant species growing in the same block (NPK or CSS) and asterisks indicate significant differences between fertilisation regimes within each plant species. Note the graphs use a different y-axis scale.

Table 3
Biomass production, Ni yield and Bioconcentration Factor (BCF, mean ± SE) for each plant species after the 10-month growth period in NPK fertilised (NPK) and composted sewage sludge (CSS) amended plots. Letters indicate significant differences between plant species and asterisks indicate significant differences between the type of fertilisation to each plant species ($p < 0.05$).

	Biomass (kg ha^{-1})	Ni yield (kg ha^{-1})	BCF × 10^2
B. emarginata			
NPK	247.3 ± 219.9 a*	2.2 ± 2.0 a	40.3 ± 16.0 a*
CSS	1468.2 ± 270.8 a	2.9 ± 0.5 a	18.3 ± 1.5 a
N. caerulescens			
NPK	82.2 ± 40.3 a*	1.5 ± 1.1 a	46.3 ± 30.7 a*
CSS	231.9 ± 31.6 b	1.9 ± 0.2 a	11.8 ± 0.9 a
O. muralis			
NPK	215.7 ± 92.9 a*	0.3 ± 0.2 a*	0.5 ± 0.2 b*
CSS	2402.0 ± 343.8 a	2.3 ± 0.8 a	15.8 ± 0.4 a
O. serpyllifolia			
NPK	194.9 ± 167.7 a*	1.0 ± 1.0 a	20.1 ± 15.6 ab*
CSS	462.3 ± 30.3 b	0.7 ± 0.1 b	0.5 ± 0.0 b

variability in plant biomass was observed amongst plant individuals of *B. emarginata* and *O. serpyllifolia* in NPK plots, and of *B. emarginata* and *O. muralis* in CSS plots (Table 3).

3.3. Effect of soil amendment on shoot Ni concentration, Ni yield and bioaccumulation

In NPK plots, shoot Ni concentrations varied significantly amongst the four plant species, but also amongst plant individuals of the same species (Fig. 3). *Bornmuellera emarginata* presented the highest shoot Ni concentrations (with a mean of 6174 ± 1692 mg kg^{-1}) and *O. muralis* the lowest (with a mean of 1140 ± 381 mg kg^{-1}). *N. caerulescens* and *O. serpyllifolia* accumulated intermediate concentrations, with mean concentrations of 3627 ± 1264 and 3520 ± 2078 mg kg^{-1}, respectively (Fig. 3). Compost addition induced a substantial reduction in shoot Ni concentration in all plant species except *O. muralis*: shoot Ni concentrations were significantly decreased by 3.3-, 2.5- and 1.8-fold in *B. emarginata*, *N. caerulescens* and *O. serpyllifolia*, respectively (Fig. 3).

A similar trend was observed for plant Ni yields, which decreased in

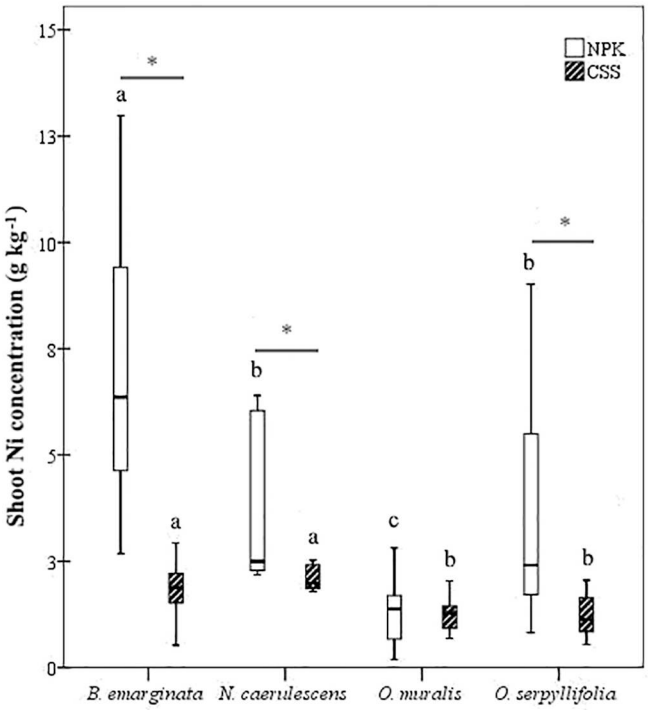

Fig. 3. Shoot Ni concentration (mean ± SE) of plants grown in the inorganic (NPK)- or compost (CSS)- amended plots. Median values are represented with a horizontal line within the boxes. Letters indicate significant differences between plant species with each fertilisation regime, and asterisks between the type of fertilisation within each plant species.

NPK plots in the following order: *B. emarginata* > *N. caerulescens* > *O. serpyllifolia* > *O. muralis* (Table 3). The mean Ni yield of *O. muralis* was increased by 7.7-fold when this species was grown in CSS plots compared to NPK plots ($p < 0.05$), reaching values equivalent to 2.3 kg Ni ha^{-1}. The same was observed, but to a lesser extent, with *B. emarginata* and *N. caerulescens*. In contrast, a 1.4-fold decrease in Ni yield of *O. serpyllifolia* was obtained when this hyperaccumulator was grown in

Table 4
Concentration of macro- and micronutrients (mean ± SE) in shoots of the four hyperaccumulating plant species of the NPK fertilised (NPK) and compost amended (CSS) plots at the harvest stage. Letters indicate significant differences between plant species in each plot (NPK or CSS) and asterisks significant differences between the effect of the type of fertilisation on each plant species ($p < 0.05$).

	Ca	K	Mg	P	Fe	Mn	Co	Cu	Zn	Ca/Mg
	($g\,kg^{-1}$)				($mg\,kg^{-1}$)					
B. emarginata										
NPK	10.3 ± 0.3 c	23.0 ± 1.1 a*	7.7 ± 0.6 b*	5.7 ± 0.2 b*	47.0 ± 27.1 c	91.1 ± 38.7 b*	11.1 ± 2.6 b	3.5 ± 0.1 b*	21.5 ± 0.1 a*	1.4 ± 0.1 b
CSS	11.1 ± 0.4 b	36.6 ± 1.4 a	6.0 ± 0.5 b	7.2 ± 0.2 b	79.3 ± 13.2 b	154.4 ± 19.6 b	15.3 ± 6.3 b	5.9 ± 0.2 c	172.2 ± 2.6 c	1.8 ± 0.1 b
N. caerulescens										
NPK	10.3 ± 0.8 c	12.8 ± 2.6 b	18.4 ± 3.0 a*	4.2 ± 0.7 c*	3658.9 ± 699.3 a*	161.3 ± 15.4 a*	18.1 ± 2.1 a*	6.4 ± 0.6 a*	19.5 ± 2.3 ab*	0.6 ± 0.1 b
CSS	10.4 ± 0.3 b	11.0 ± 1.1 d	11.0 ± 3.5 a	7.3 ± 1.0 b	1644.4 ± 971.3 a	294.4 ± 59.6 a	29.8 ± 8.4 a	18.3 ± 2.7 a	1564.7 ± 275.1 a	1.1 ± 0.3 c
O. muralis										
NPK	13.6 ± 0.7 b*	12.8 ± 0.7 b*	3.4 ± 0.4 c	3.5 ± 0.2 c*	125.3 ± 33.5 b	78.4 ± 8.3 c*	4.9 ± 0.8 d*	5.8 ± 0.4 a*	15.6 ± 6.7 b*	4.1 ± 0.5 a
CSS	11.4 ± 0.1 b	25.5 ± 0.3 b	2.3 ± 0.2 c	4.8 ± 0.2 c	103.7 ± 22.8 b	263.8 ± 10.0 a	30.7 ± 2.1 a	12.6 ± 0.8 b	152.3 ± 2.4 c	4.9 ± 0.4 a
O. serpyllifolia										
NPK	18.4 ± 1.0 a*	10.5 ± 0.7 b*	7.5 ± 0.4 b*	6.4 ± 1.3 a*	178.4 ± 14.8 b*	84.9 ± 10.3 bc*	8.2 ± 0.5 c	3.0 ± 0.6 b*	20.3 ± 2.2 a*	2.5 ± 0.0 a*
CSS	14.4 ± 0.1 a	16.4 ± 2.0 c	4.7 ± 0.1 b	12.4 ± 1.1 a	99.5 ± 24.8 b	123.6 ± 18.5 b	8.8 ± 2.9 b	5.5 ± 0.1 c	305.8 ± 11.5 b	3.0 ± 0.1 b

Table 5
Colony forming units per gram fresh soil (CFUs * $10^6\,g^{-1}$ ± SE) in planted and non-planted soils of the NPK fertilised (NPK) and the compost amended (CSS) plots and the ratio planted to non-planted plots (P/NP). Letters indicate significant differences between planted or non-planted sub-plots under the same fertilisation regime and asterisks indicate differences between the type of fertilisation (NPK or CSS) to each plant planted or non-planted sub-plots ($p < 0.05$).

	284		Ni 2.5 mM	
	CFUs * 10^6	P/NP	CFUs * 10^6	P/NP
Non-planted				
NPK	1.9 ± 0.1 b		0.1 ± 0.0 b	
CSS	2.7 ± 0.2 b		0.1 ± 0.0 b	
Bornmuellera emarginata				
NPK	5.4 ± 2.7 b	2.8 a	0.6 ± 0.1 ab	5.2 b*
CSS	10.2 ± 2.4 a	3.7 b	0.9 ± 0.2 ab	8.5 b
Noccaea caerulescens				
NPK	11.2 ± 1.7 a*	5.8 a*	0.7 ± 0.0 a*	5.7 b*
CSS	19.1 ± 3.6 a	7.0 a	1.7 ± 0.2 a	15.2 a
Odontarrhena muralis				
NPK	7.0 ± 2.0 a*	3.6 a	1.2 ± 0.8 a*	10.1 a*
CSS	12.3 ± 2.8 a	4.5 b	0.3 ± 0.1 b	3.2 b
Odontarrhena serpyllifolia				
NPK	7.7 ± 1.9 a*	4.0 a*	1.2 ± 0.0 a	10.2 a
CSS	26.8 ± 2.9 a	9.8 a	1.7 ± 0.7 a	15.6 a

CSS plots ($p > 0.05$; Table 3).

Finally, *B. emarginata*, *N. caerulescens* and *O. serpyllifolia* presented elevated BCF values when grown in the NPK-fertilised plots, ranging from 20.1 (*O. serpyllifolia*) to up to 46.3 (*N. caerulescens*). In contrast, BCF values for these species were 2.2-, 3.9- and 20.1-fold lower when grown in compost-amended plots (Table 3). The opposite was observed in the case of *O. muralis*: the BCF of this species was 31.6-fold higher when grown in compost-amended soil compared to NPK fertilisation (Table 3).

3.4. Effect of soil amendment on plant nutrients

Some differences in shoot ionome were observed between plants grown in soils amended with compost or fertilised with inorganic NPK (Table 4). Compost amendment led to significant increases in shoot P and K concentrations in all plant species (except for *N. caerulescens* in the case of K) compared to NPK fertilisation ($p < 0.05$). In contrast, shoot Ca concentration was reduced by 1.2- and 1.3-fold in *O. muralis* and *O. serpyllifolia* ($p < 0.05$) in CSS plots compared to NPK plots, whereas no differences in this element were observed in *B. emarginata* and *N. caerulescens*. All plant species except *O. muralis*, presented significantly lower shoot Mg concentrations in CSS plots ($p < 0.05$). In accordance, amending with compost tended to increase the shoot Ca/Mg quotient, although this was only significant for *O. serpyllifolia* ($p < 0.05$). The *Odontarrhena* species presented the highest Ca/Mg quotient in both NPK and CSS plots.

A generalized effect of compost amendment on shoot micro-nutrient concentrations was observed in all four hyperaccumulators (Table 4). Shoot Mn, Cu and Zn concentrations were consistently and significantly higher in all plants grown in CSS plots, and shoot Co concentration in shoots of *N. caerulescens* and *O. muralis* ($p < 0.05$). On the other hand, the compost amendment tended to reduce shoot Fe concentration in *N. caerulescens*, *O. serpyllifolia* and *O. muralis* (significant in case of the first two species). In CSS plots, *N. caerulescens* presented notably higher concentrations of Fe and Zn than any of the other three species (Table 4). For example, a mean concentration of 1564 ± 276 mg Zn kg^{-1} was recorded, whereas in the other species this ranged from 152 ± 2 mg Zn kg^{-1} in *O. muralis* to 31 ± 12 mg Zn kg^{-1} in *O. serpyllifolia*.

Table 6
Average well colour development (AWCD), Shannon's diversity (H′), Shannon's evenness (J′) and richness (S) indices. Letters indicate significant differences amongst plant species under the same fertilisation regime (NPK or CSS) and asterisks significant differences between inorganic and organic amendment on each planted or unplanted sub-plots ($p < 0.05$).

	AWCD	H′	J'	S
Non-planted				
NPK	0.61 ± 0.01 b *	3.38 ± 0.01 b*	0.73 ± 0.02 b*	25.0 ± 1.3 b*
CSS	2.06 ± 0.02 b	4.84 ± 0.02 a	1.02 ± 0.03 b	27.0 ± 1.2 a
B. emarginata				
NPK	1.69 ± 0.09 a *	4.69 ± 0.12 a	0.99 ± 0.03 a	27.0 ± 3.0 a
CSS	2.31 ± 0.04 a	4.93 ± 0.01 a	1.02 ± 0.00 a	29.3 ± 1.3 a
N. caerulescens				
NPK	1.64 ± 0.16 a *	4.87 ± 0.19 a	1.01 ± 0.02 a	29.0 ± 1.2 a
CSS	2.32 ± 0.10 a	4.91 ± 0.01 a	1.02 ± 0.00 a	29.0 ± 1.5 a
O. muralis				
NPK	1.87 ± 0.15 a	4.68 ± 0.09 a	0.95 ± 0.09 a	27.7 ± 1.7 a
CSS	2.16 ± 0.03 ab	4.93 ± 0.05 a	1.00 ± 0.01 a	29.0 ± 1.7 a
O. serpyllifolia				
NPK	1.40 ± 0.02 a *	4.60 ± 0.10 a*	0.97 ± 0.03 a	27.0 ± 1.0 a
CSS	2.30 ± 0.03a	4.93 ± 0.01 a	1.01 ± 0.01 a	29.3 ± 1.3 a

3.5. Effect of soil amendment on soil microbial parameters

The densities of culturable bacteria associated with the four hyperaccumulators and non-planted (NP) soils are presented in Table 5. Bacterial densities in NP soil did not differ significantly between NPK-fertilised and compost-amended plots, ranging from 1.9×10^6 CFUs g^{-1} soil in the NPK plot to 2.7×10^6 CFUs g^{-1} soil in the CSS plot. In these soils, Ni-tolerant bacteria represented 6.0 and 4.0% of the total culturable population, respectively. All plant species grown in CSS plots harboured a higher bacterial density compared to the same species in NPK plots ($p < 0.05$). In addition, a plant-species specific effect was observed in both NPK and CSS plots: *N. caerulescens* and *O. serpyllifolia* harboured the highest bacterial densities whereas the lowest were observed for *B. emarginata*. The plant-induced stimulation in bacterial densities is also reflected by the P/NP ratios (Table 5). In addition, planted soils (both in NPK and CSS plots) harboured higher densities of Ni-tolerant bacteria compared to NP soils, although this increase was not always significant. The *Odontarrhena* species harboured the highest proportion of Ni-tolerant bacteria when grown in NPK plots, representing 16.6% and 15.1% of the total culturable population, followed by *B. emarginata* (10.8%). However, the same was not observed in CSS plots, where the proportion of Ni-tolerant bacteria in relation to the total culturable population was lower, especially in the case of *O. muralis* (3.5%).

The overall functional activity of the soil heterotrophic microbial community was evaluated by FDA activity and the AWCD values obtained from Biolog Ecoplates. Compost-amended soils presented significantly higher microbial activity (on the basis of AWCD) in both planted (except *O. muralis*) and NP soils compared to corresponding NPK-fertilised soils (Table 6). Mean AWCD in NP plots increased by 3.3-fold in the presence of organic amendment, while in planted plots this increase ranged from 1.2- to 1.6-fold. Within planted soils, AWCD did not differ significantly amongst the four hyperaccumulating plant species. The Shannon's diversity (H′), Shannon's evenness (J′) and richness (S) indices are given in Table 6. Compost amendment led to an increase in H′ and J′ values in comparison with inorganic fertilisation, and this was significant for NP soils (both H′ and J′), and for *O. serpyllifolia* (H′). Also, increases in H′ values were observed in planted soils, and this was especially pronounced in NPK plots. The same trend was observed in evenness (J′) values obtained in NPK plots. No significant differences were observed amongst the different plant species for either H′ or J′ indices.

In addition to the increase in AWCD, a higher number of C substrates were utilised in CSS plots compared to NPK plots (S values, $p < 0.05$; Table 6). Plant growth also stimulated the functional diversity of the soil microbial communities, as reflected by the higher numbers of metabolised C substrates in planted soils compared to NP soils. This plant-induced effect was observed in both NPK and CSS plots. However, few differences in S values were observed amongst the four plant species.

Fig. 4 shows the substrate average colour development (SAWCD) of the microbial community associated with the different plant species and NP soils. SAWCD values were highest for carbohydrates, amines and polymers in both NPK and CSS plots, corresponding with more than 50% of the metabolised C sources. Amending with compost led to shifts in the C source utilisation patterns. SAWCD values were higher in compost-amended soils (both NP and planted) compared to NPK-soils, and this was particularly pronounced for amines and in some cases carboxylates ($p < 0.05$). A pronounced difference in SAWCD values was observed between NP and planted soils in the NPK plots, irrelevant of the species. For example, a significant increase in the use of amines was observed in the presence of all four hyperaccumulators, and in carboxylates in the presence of *B. emarginata* and *O. muralis* (Fig. 4). In contrast, with a few exceptions C type usage patterns were similar for both planted and NP soils of the CSS plots (Fig. 4). In these plots, a significant increase in the metabolism of amines was observed after growth of the hyperaccumulators *B. emarginata* and *N. caerulescens* (Fig. 4).

Likewise, FDA hydrolase activity was significantly higher in compost-amended soils compared to NPK soils, and this was the case for both planted and NP soils ($p < 0.05$) (Fig. 5). In some cases, plant growth led to further increases in FDA activity compared to NP sub-plots ($p < 0.05$). A mean FDA activity of 5.2 ± 0.1 μg g^{-1} soil h^{-1} was recorded in NP soils of the NPK plots, and this increased to 9.6 ± 0.5 μg g^{-1} soil h^{-1} and 18.9 ± 0.1 μg g^{-1} soil h^{-1} in soils under *N. caerulescens* and *O. muralis*, respectively ($p < 0.05$). In CSS plots, FDA activity was stimulated in soils under *B. emarginata* and *N. caerulescens*, increasing from a mean value of 29.7 μg g^{-1} soil h^{-1} in NP soils to 42.5–45.7 μg g^{-1} soil h^{-1} (Fig. 5).

4. Discussion

The mine spoil soil was deficient in essential nutrients and organic matter, and presented high concentrations of Ni, Mn and Cr, as was previously shown for this site (Ghasemi et al., 2018a). Compared to the natural serpentine soils of the Melide ultramafic complex, which are in the same region as the quarry (Álvarez-López et al., 2016b; Ghasemi et al., 2018b), the mine-soil pH is significantly higher, while the C/N ratio and CEC are lower. Nutrient availability, including P and K, are also minimal. On the other hand, the presence of carbonates in the

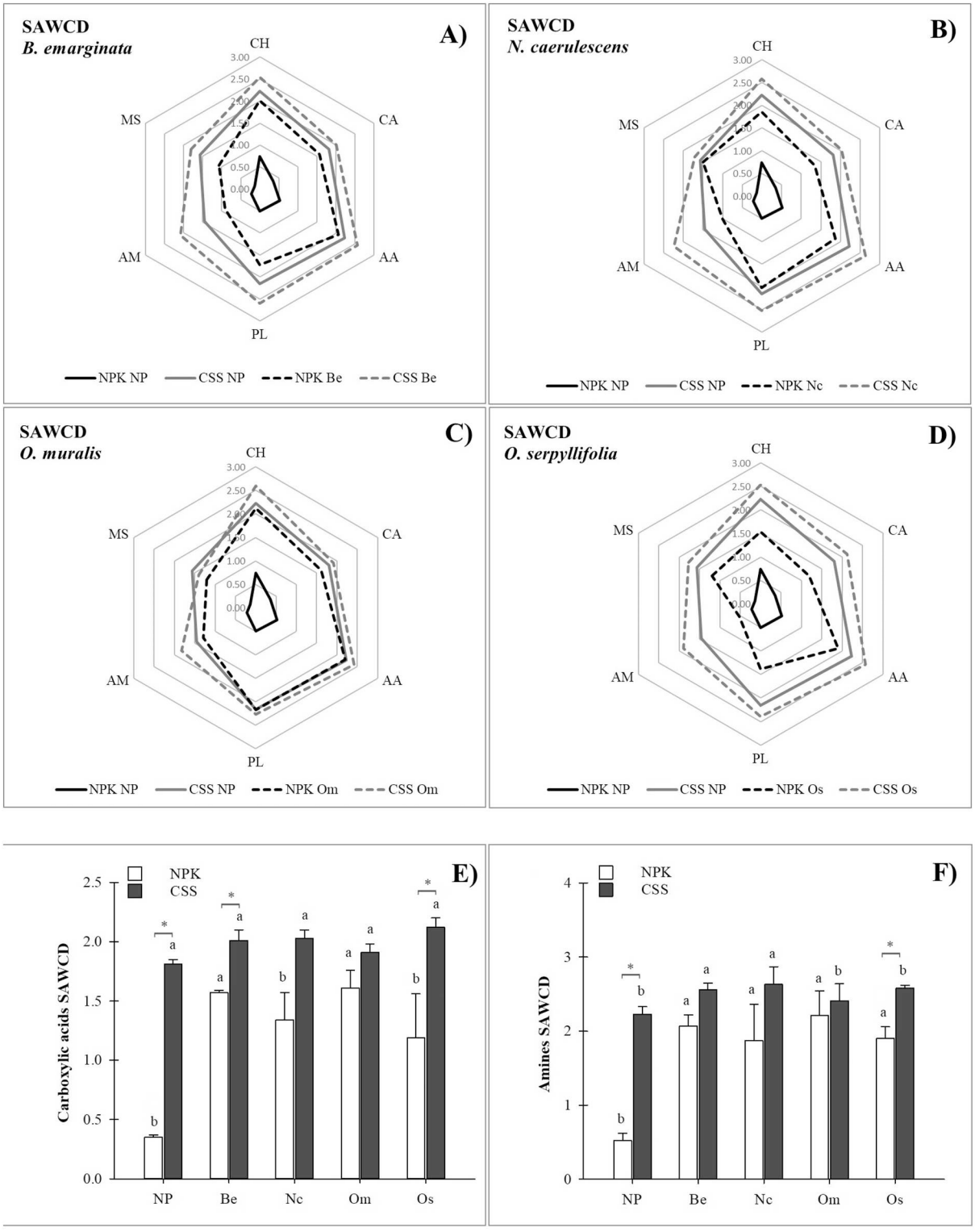

Fig. 4. Substrate average well colour development (SAWCD) in non-planted and planted soils of the inorganic (NPK)-fertilised and compost (CSS)-amended plots. NP: non-planted, Be: *B. emarginata*, Nc: *N. caerulescens*, Om: *O. muralis*, Os: *O. serpyllifolia*; CH: carbohydrates, CA: carboxylic acids, AA: amino acids, PL: polymers, AM: amines, MS: miscellaneous.

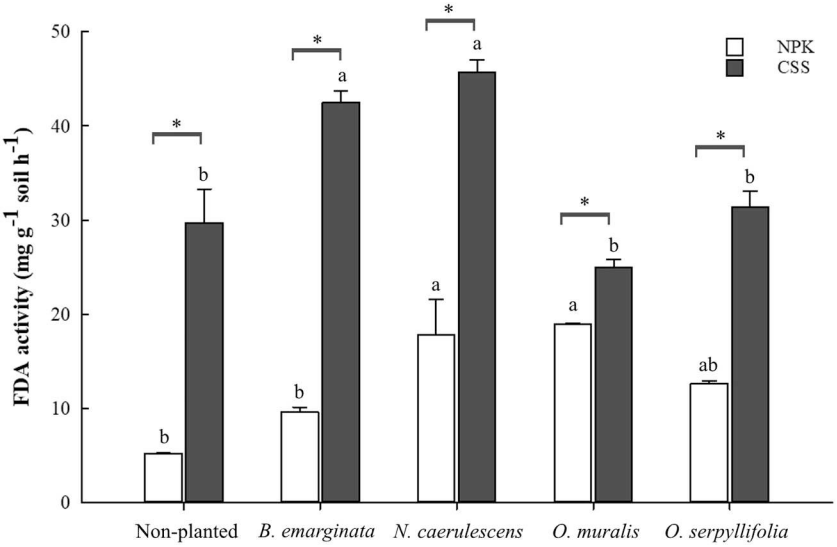

Fig. 5. FDA activity in planted or non-planted soils of the inorganic (NPK)- and compost (CSS)-amended plots (mean ± SE). Letters indicate significant differences between plant species or non-planted soils under the same fertilisation regime and asterisks significant differences between the type of fertilisation within each plant species or non-planted soil ($p < 0.05$).

parent material results in a more balanced Ca:Mg ratio than that usually found in these natural serpentine soils. Although the pseudo-total Ni concentrations are comparable to those recorded in the natural serpentine soils from the region, Ni availability in the spoil (estimated using DTPA-extractable concentrations) is up to two magnitudes lower (mean DTPA-extractable Ni was 3.8 mg kg^{-1} compared to close to 100 mg kg^{-1}; Ghasemi et al., 2018b).

Improving the physical, chemical and biological properties of the mine-soil is necessary for plant establishment and development. The improvement in soil fertility through the addition of inorganic NPK fertilisers has been shown to be effective for enhancing the growth of hyperaccumulating plants in natural serpentine soils (*Bani* et al., 2015a; Li et al., 2003), but organic amendments can also overcome soil physical deficiencies such as the poor structure and porosity and limited water retention capacity (Álvarez-López et al., 2016b; Ghasemi et al., 2018b) and positively influence soil microbial activity (Benbi et al., 2017). Here, the addition of the composted sewage sludge also improved mine-soil pH for hyperaccumulator growth. At the end of the field experiment, pH values in the unplanted CSS plots were within the optimal range defined for Ni phytoextraction by *Odontarrhena* species (pH 5–6.5) (Nkrumah et al., 2016) and were maintained after plant growth with no significant differences amongst plant species. The improvements in soil fertility (increase in P availability and exchangeable Ca and K) were reflected in the shoot ionome of plants growing in CSS plots. Shoot concentrations of most macro- and micro-nutrients were in the range found for *Odontarrhena* spp., *Noccaea* spp. and *B. emarginata*, growing in Mediterranean ultramafic areas (Bani et al., 2010) or natural serpentine soil from the quarry region (Álvarez-López et al., 2016a; Pardo et al., 2018). In contrast, shoot Fe concentrations decreased in all the hyperaccumulating species (except *B. emarginata*) in the CSS plots, which could be the result of complexation with the organic matter (Lützow et al., 2006). It is worth noting the elevated shoot Fe concentrations found in *N. caerulescens* when grown in both NPK and CSS plots, which may reflect a high capacity of this population to bioaccumulate Fe.

DTPA-extractable concentrations of Cu, Mn, Ni and Zn were significantly higher in the CSS plots compared to NPK plots. In the case of Cu, Mn and Zn, these elements originate from the compost itself (Table 1), while this is not the case for Ni (total Ni concentrations in the compost were < 40 mg kg^{-1}). However, in all cases further increases in the availability of these metals were observed after plant growth. These results are in agreement with those of Ghasemi et al. (2018a) who used the same mine-soil in a pot experiment with different *Odontarrhena* spp. (including *O. serpyllifolia*). However, they contrast with other authors who have found significant reductions in the Ni availability in natural serpentine soils after amendment with composted sewage sludge (the same compost as this study) (Álvarez-López et al., 2016a). The increase in Ni availability after the incorporation of compost was likely due to the weathering activity of labile C compounds (e.g. carboxylic acids) present in the compost on the Ni-rich minerals and release of labile Ni. Several authors have shown an increase in soil metal availability in the rhizosphere of hyperaccumulating plants, such as those belonging to the genera *Odontarrhena* (Ghasemi et al., 2018b; Kidd et al., 2009) and *Noccaea* (Puschenreiter et al., 2005; Wenzel et al., 2003). This is generally related to the presence of carboxylic acid anions (such as oxalate) or simple phenolics present in the root exudates which are released into the rhizosphere (Kidd et al., 2009). Chardot-Jacques et al. (2013) found that the growth of the Ni-hyperaccumulator *Leptoplax emarginata* (syn. *B. emarginata*) increased the dissolution of chrysotile, a silicate from the serpentine group with low Ni availability. These authors suggested that the high Ni uptake by the plant causes a decrease in water-soluble Ni which in turn induces chrysotile dissolution. More recently, these phenomena have been connected to the activity of plant-associated bacteria on metal speciation and bioavailability (Kidd et al., 2017).

Pseudo-total Zn concentrations in the mine-soil were in the range for normal soils (Kabata-Pendias and Pendias, 2001) but were increased by 6.2-fold after the addition of the compost, and DTPA-extractable Zn concentrations were also 45.7-fold higher. *Noccaea caerulescens* presented particularly elevated shoot Zn concentrations in these plots (mean concentration of 1565 mg Zn kg^{-1}) compared to the other plant species (one order of magnitude higher). Individuals of this population of *N. caerulescens* growing naturally in the surrounding area of the quarry also show elevated Zn accumulation (shoot concentrations ranging from 320 to 1450 mg Zn kg^{-1}) at the same time as Ni hyperaccumulation (4500–18,700 mg Ni kg^{-1}), despite low pseudo-total and available Zn concentrations (< 100 mg kg^{-1}; unpublished results). In addition, growth of *N. caerulescens* (together with *O. muralis*) led to the highest stimulation in soil Zn availability. Other authors have found that several *Noccaea* spp., including *N. caerulescens*, are able to accumulate Zn from Zn-rich soils but also from soils containing "normal" concentrations of this element (Bani et al., 2010; Reeves et al., 2001). It would be interesting to further evaluate the Zn accumulation capacity of the population of *N. caerulescens* used in this study in Zn-enriched soils.

Compared to natural serpentine soils in this region, culturable bacterial densities and metabolic activity and diversity of the mine-soil microbial community was low (Álvarez-López et al., 2016a; Pardo

et al., 2018), confirming the unfavourable conditions for microbial growth in these substrates. The input of nutrients and labile C present in the compost stimulated the microbial activity and diversity. These results coincide with previous studies showing improvements in soil physico-chemical and biological properties of mine-soils after addition of organic residues (Garaiyurrebaso et al., 2017; Pardo et al., 2014). As expected, planted soils always harboured higher bacterial densities than NP soils. This plant effect is likely due to the release of labile C and growth factors present in the root exudates, which was generally more pronounced in CSS plots where plant root growth and proliferation were enhanced. *Noccaea caerulescens* and *O. serpyllifolia* harboured higher bacterial densities under both fertilisation regimes (highest P/NP ratios). This could partly be attributed to the fact that these plant species are naturally found growing in natural serpentine soils of the region, whereas the other two plant species are native to the Mediterranean region. It is well recognised that the size and composition of bacterial communities associating with hyperaccumulators is plant species-specific but also differs amongst different populations belonging to the same species (Álvarez-López et al., 2016a). The incorporation of the amendment and the presence of plants had a strong effect on the overall functional activity of soil microbiota, as assessed with the Biolog-CLPP parameters and FDA activity. The AWCD and FDA values were increased by 3- to 6-fold in NP soil of CSS plots compared to NPK plots. Plants led to further increases in both soil microbial densities and activity. The FDA activity was substantially higher in the soil under *B. emarginata* and *N. caerulescens*, despite the lower bacterial densities associated with the former plant species. Increased soil hydrolytic capacity has been related to a higher soil C content and number of metabolically active microorganisms (Benbi et al., 2017). In agreement, the soils associated with these two plant species also showed the highest C content as well as AWCD values. Furthermore, a generalized effect of all plant species was found on the number of C substrates utilised (the metabolic richness, S) and the diversity in the use of these C substrates (indicated by the H′ index). The presence of elevated concentrations of metals in contaminated or metalliferous soils exerts selective pressure on soil microorganisms, and strongly influences the composition of the microbial community (Burges et al., 2016; Harantová et al., 2017). The addition of the compost may have a protective effect on the microorganisms from the unfavourable abiotic conditions of the mine-soil, which could explain the decrease in densities of Ni-tolerant bacteria in the CSS plots compared to NPK plots. However, despite the higher proportion of Ni-resistant bacteria in NPK soils, the compost-amended soil also showed elevated P/NP ratios (except for *O. muralis*). Several authors have found a positive correlation between the presence of hyperaccumulator plants and the bacterial density or relative gene abundance of metal-resistant bacteria in soil; this was the case for *N. caerulescens* and *O. serpyllifolia*, and for various metals, including Ni, Zn and Cd (Álvarez-López et al., 2016a; Epelde et al., 2010; Mengoni et al., 2010). Since plant-associated bacteria can influence metal mobility and availability in soils, as well as promote a higher tolerance and biomass production, studying the microbial communities in such mine spoils could be useful for identifying bioinoculants within the context of optimising the Ni phytomining strategy.

Plant survival varied considerably amongst replicate plots of the same species and under the same fertilisation regime. Surprisingly, it was not significantly enhanced after compost addition (with the exception of *B. emarginata*). Data obtained from the nearest meteorological station (Lalín, NW Spain) showed that the Spring and Summer of 2017 was characterised for being extremely warm and dry. Registered precipitation in April was 83% lower than the normal values for this region and season. In May, maximal temperatures of 36.2 °C were recorded, with daily oscillations of up to 30.7 °C. During the whole experimental period (September 2016 to July 2017) the mean temperature was 11.7 °C, with a fluctuation between +37.4 °C and − 6.5 °C, and an accumulated precipitation of 819 l m^{-2}. In accordance, the plants were affected both by late frosts and extreme drought, which accounts for the high mortality rates in some species. Unexpectedly, the highest plant mortality was observed for the native *O. serpyllifolia*, which is adapted to the natural serpentine soils in this region: plant survival did not reach 50% in either the NPK or CSS plots for this species. In greenhouse pot experiments, despite limited growth, no mortality was observed in this species when grown in the same mine-soil (Ghasemi et al., 2018a).

Nonetheless, the positive effects of compost addition on soil quality (discussed above) were clearly reflected in the stronger growth and healthier aspect of all plant species in the CSS plots. Plants responded dramatically to organic amendment with greatly improved shoot DW yields. Best response in terms of biomass production was obtained for the hyperaccumulators *O. muralis* and *B. emarginata*, where the mean biomass yields were equivalent to 2402 and 1468 kg ha^{-1}, respectively. Both these Mediterranean plant species have been recognised for their potential application in nickel agromining in Albanian ultramafic soils (van der Ent et al., 2015). This study suggests that they are also good candidates for developing phytomining activities in degraded Ni-enriched mine-soils. However, in contrast to this positive response in biomass production, amending the mine-soil with compost led to significant reductions in shoot Ni concentrations. This was the case for all plant species except *O. muralis*, which reinforces the suitability of this species in the phytomining technique. Similar reductions in shoot Ni concentrations have been observed in greenhouse studies for several hyperaccumulating plant species when grown in natural serpentine soils amended with organic matter (Álvarez-López et al., 2016b) but also in spoil soils from the same quarry as the present study (Ghasemi et al., 2018a). These authors also observed a reduction in the BCF values after compost addition in several species of *Odontarrhena* (including *O. serpyllifolia*) and *Noccaea*; in all cases the reduction in BCF was proportional to the rate of addition. In this study, an increase in the BCF after compost addition was only observed for *O. muralis*, confirming the higher capacity of this species to extract and accumulate Ni in the shoots compared to the other plant species. The reduction in shoot Ni concentration could be due to a dilution effect since the promotion in plant biomass was not mirrored by a reduction in soil Ni availability (DTPA-extractable).

Despite the reductions in shoot Ni concentrations observed in *B. emarginata* and *N. caerulescens* grown in the compost-amended plots, the stimulation in shoot DW biomass led to significantly higher Ni yields. In the case of *O. serpyllifolia*, although the biomass of this species was enhanced with the compost the shoot Ni concentration was strongly reduced, resulting in a lower Ni yield in the CSS soil. Finally, shoot Ni concentrations of *O. muralis* were the lowest compared to the other three plant species; however, due to the strong response in this species to compost addition the overall Ni yield was close to that obtained with *B. emarginata*. Similarly, Chardot et al. (2005) found in a pot experiment using three different soils that *B. emarginata* accumulated Ni concentrations as high as *N. caerulescens* and *O. muralis* but produced a higher biomass. In a one-year agromining trial using these two hyperaccumulating plant species, and carried out in natural soils of the same serpentine outcrop as the quarry, the total biomass harvested was 996 kg ha^{-1} and 679 kg ha^{-1} for *O. muralis* and *B. emarginata*, respectively (Pardo et al., 2018). The biomass production at the quarry site was therefore > 2-fold higher than that obtained in these natural soils. Although the authors of the latter study pointed out that the biomass yields obtained were low due to adverse climate and growth conditions: elevated precipitation which resulted in soil waterlogging, together with competition from proliferating weeds, led to significant plant mortality. In contrast, in the study of Pardo et al. (2018), shoot Ni concentrations of *O. muralis* were 3.8- and 3.2- fold higher than those obtained for *O. muralis* in the NPK and the CSS plots, respectively, and 0.7- and 2.2-fold higher in the case of *B. emarginata* (in NPK and CSS, respectively). Bani et al. (2015a) conducted a field study evaluating the performance of *O. muralis* for extensive phytomining in the ultramafic area of Pojskë (Pogradec, Albania) and obtained both biomass and Ni

yields considerably higher than this study. This can be explained by the significantly lower soil Ni availability in the quarry experimental site compared to the Albanian ultramafic soils. Moreover, the results given by these authors were obtained after five years of optimising the cropping patterns and fertilisation regimes. Given that the results presented here are based on one single growth season, the efficiency of the system is expected to be progressively improved in subsequent growth cycles. The sub-optimal shoot K concentrations found in some plots and the elevated Cu and Zn concentrations in the compost may also have impaired plant growth and Ni accumulation of the study species. The severe drought conditions experienced affected plant survival, and the implementation of an irrigation system at the site is expected to help overcome this problem. Considering a plant survival rate of 100%, the potential biomass yields for *B. emarginata*, *N. caerulescens*, *O. muralis* and *O. serpyllifolia* would be 293, 116, 295 and 291 kg ha^{-1}, respectively, in NPK plots, and 1951, 385, 5016 and 1069 kg ha^{-1}, respectively, in the CSS plots. The corresponding potential Ni yields would be 2.5, 2.0, 0.6 and 2.2 kg ha^{-1}, and 3.7, 3.1, 8.3 and 3.2 kg ha^{-1}, in the NPK and CSS plots, respectively. Further improvements in biomass and Ni yields are required if the process is to be economically viable, such as that reported by Bani et al. (2015a). Improvements could be achieved through agronomic practices, such as plant cropping patterns (intercropping with legumes) or increasing plant densities. For example, in the case of *B. emarginata* which showed a high survival rate, a plant density of 4 plants per m^2 did not completely cover the soil surface and higher Ni yields may be obtained by optimising planting densities for this species. In the case of *N. caerulescens*, a higher planting density (81 plants/m^2) was used due to its small size; however, this density successfully covered the full surface area (in those plots where survival was high). Jacobs et al. (2018) found that the biomass production of *N. caerulescens* at a density of 100 plants m^2 was highest but not double that obtained with 50 plants m^2, indicating competition for resources, such as nutrients, water or light. However, no competition for metals was observed and plants showed similar shoot metal concentrations between the different planting densities. The authors suggested that the plants were forced to explore a wider volume of soil when grown at a high density. Planting density exerts a strong effect on plant growth and yield, and shoot metal concentration (Bani et al., 2015b; Li et al., 2003), and therefore needs to be assessed for each plant species and experimental site. In the case of *N. caerulescens*, the high number of plants required per unit surface area and its low biomass production make this plant species less appropriate for phytomining application.

5. Conclusions

To the best of our knowledge this is the first field study carried out which assesses Ni phytomining viability in serpentine mine-soils in the Iberian Peninsula. Clearly, plant species selection and further development of the technique is necessary to fully optimise plant survival, biomass production and Ni yields. Overall, the Mediterranean hyperaccumulators *B. emarginata* and *O. muralis* showed an extraordinary capacity to adapt to the unfavourable conditions of the mine-soil under the humid-temperate climate. On the other hand, the native hyperaccumulator *O. serpyllifolia* showed the lowest rates of survival in both NPK- and CSS-amended plots. Compost addition was more effective than NPK fertilisation and, together with plant growth, led to significant improvements in soil quality in terms of physico-chemical and biological properties. Amending with compost increased the total amount of Ni phytoextracted (the Ni yield), and this was mainly driven by the stimulation in biomass production rather than an increase in Ni uptake by the hyperaccumulating plants. The dilution effect observed regarding shoot Ni accumulation in the presence of compost, as well as the presence of potentially phytotoxic metals in this amendment, could limit both plant growth and Ni phytoextracted. Further studies should focus on the appropriate selection of the type of the organic amendment used, as well as the optimal addition rate, for increasing soil Ni availability and enhancing its uptake by hyperaccumulating plants while maximising plant biomass production. Overall, the results confirm the potential for phytomining to be part of a rehabilitation strategy during mining operations and after the closure of the mine.

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